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14. ABSTRACT The high level of exposure of our troops to UV radiation increases their risk of melanoma, the deadliest form of skin cancer. The major genotype in melanoma are mutant BRAF (40-60%) and mutant NRAS (15-20%). Significant advances have been made in the understanding of mutant BRAF melanoma leading to the FDA-approval of the RAF inhibitor PLX4032/Vemurafenib/zelboraf. By contrast, mutant NRAS melanomas are non-responsive to Vemurafenib. Hence, treatment options for mutant NRAS melanomas are reliant on standard chemotherapies and immunotherapies, which have low response rates and high toxicity. Directly targeting RAS has not proven to be clinically effective in RAS-driven tumors. In this proposal, we will focus on downstream RAS effectors. Furthermore, since resistance to monotherapies is evident in melanoma and other tumor types, we will apply a combinatorial approach to simultaneous block multiple arms of mutant NRAS-mediated signaling. Specifically, we hypothesize that TANK-binding kinase 1 (TBK1) mediates cell survival in mutant NRAS melanomas and that targeting TBK1, in combination with the distinct RAS-regulated MEK-ERK1/2, will promote cytotoxicity and reduce mutant NRAS melanoma growth. In sum, this proposal will utilize an innovative *in vivo* reporter system and aims to identify novel mechanisms of resistance in melanoma. At the completion of the proposed experiments, the identification of novel resistance-promoting mechanisms in mutant B-RAF melanomas will have a profound impact on future RAF inhibitor clinical trial strategies for both military personnel and the general public.

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Introduction

The high level of exposure of our troops to UV radiation increases their risk of melanoma, the deadliest form of skin cancer. Melanoma is characterized by frequent V600E mutations in the serine-threonine kinase, BRAF. In a phase 1 trial, a BRAF inhibitor, PLX4032/RG7204, elicited objective initial responses in 81% of melanoma patients harboring BRAF mutations [1]. Response correlates closely with >80% inhibition of ERK1/2 activity in patients [2]. However, most patients that initially responded have subsequently relapsed indicating acquired resistance. The mechanisms underlying resistance to BRAF inhibitors are unknown and must be elucidated to optimize future clinical trials. This grant aimed to utilize *in vivo* ERK1/2 activity imaging to quantify effects of BRAF inhibitors in melanoma xenografts and test for ERK1/2 pathway re-activation following tumor resistance to PLX4720 (the pre-clinical tool compound for PLX4032). Second, the grant tested for novel mechanisms of resistance to PLX4720 by molecularly modulating ERK1/2 pathway components in melanoma cells and measuring resistance to PLX4032/4720 in physiologically relevant *in vitro* and *in vivo* models. Specifically, it aimed to analyze the role of mutant NRAS and the ERK1/2 pathway scaffold protein, SHOC2, in mediating resistance to PLX4032 (now known as vemurafenib).

Body

Recently, the FDA approved the RAF inhibitor, vemurafenib (PLX4032), in late-stage, mutant BRAF melanomas. Vemurafenib was developed from a structure-guided approach to select for compounds that show selectivity towards V600E mutant forms of BRAF compared to wild-type BRAF [2]. However, the clinical response to vemurafenib is heterogeneous potentially due to variable ability to inhibit ERK1/2 activation [1,3,4]. Notably, patients with effective responses typically showed over 80% inhibition of phosphorylated ERK1/2, as judged by immunohistochemical staining [2]. A second concern is that tumors in patients, who initially show a partial or complete response, typically progress. This acquired resistance to vemurafenib is associated with re-activation of the ERK1/2 pathway by mechanisms involving mutations in NRAS and expression of BRAF splice variants [5,6]. These findings underscore the importance of monitoring the effects of RAF (and MEK) inhibitors on ERK1/2 activity in a quantitative and temporal manner in vivo. To this end, in this grant we developed an ERK1/2 activity reporter in melanoma cell xenografts to measure PLX4720 inhibition of mutant BRAF-MEK-ERK1/2 signaling.

Task 1: To utilize an ERK1/2 activity reporter in melanoma cell xenografts to measure PLX4720 inhibition of B-RAF-MEK signaling and the extent to which pathway re-activation is associated with the acquisition of resistance to PLX4720 and tumor re-growth.

i) Generate a lentiviral-based Gal4-Elk-1 reporter system in WM793, WM115 melanoma cells.

To develop a stable, ERK1/2-responsive reporter system, mutant BRAF melanoma cells WM115TR and 1205LuTR were transduced with a lentivirus carrying an EGFP-firefly luciferase fusion gene under the control of a minimal promoter with 10 tandem copies of the GAL4 upstream activation sequence (UAS). Tet repressor-expressing cells were utilized to permit future molecular-based approaches. Cells were simultaneously transduced with virus containing renilla luciferase under constitutive control from the human ubiquitin C (UbC) promoter. Renilla luciferase serves as an internal control for firefly luciferase activity *in vitro* and a measure of tumor cell biomass *in vivo*. The UbC promoter was selected as it was unresponsive to BRAF/MEK inhibition and demonstrated strong, constitutive expression in melanoma cells. The resulting cells, termed WM115TR Ubc/RL-UAS(10x)/EGFP-Luc and 1205LuTR Ubc/RL-UAS(10x)/EGFP-Luc, exhibited virtually no firefly luciferase activity relative

to renilla activity. To make firefly expression ERK1/2 responsive, cells were transduced with UbC-driven GAL4-ELK1, a fusion protein with transcriptional activity that is dependent upon phosphorylation by ERK1/2 [7,8]. The resulting cells exhibited high firefly luciferase activity, consistent with activated ERK1/2 in mutant BRAF melanoma cells [9]. Ultimately, to ensure maximal detection of firefly luciferase for *in vivo* studies, 1205LuTR Ubc/RL-UAS(10x)/EGFP-Luc-GAL4-ELK1 cells were sorted for the highest expression of EGFP.

ii) Validation of ERK reporter system *in vitro*.

To determine responsiveness to BRAF inhibition, 1205Lu and WM115 reporter cells were treated for 24 hours with PLX4720. Both cell lines showed a dramatic reduction in the firefly luciferase activity relative to renilla luciferase (Fig. 1A). Additionally, 1205LuTR cells showed a significant reduction in firefly luciferase activity in response to the MEK1/2 inhibitors, AZD6244 (selumetinib) and GSK1120212 (trametinib) (Fig. 1B). Firefly luciferase was both V5 and GFP tagged, whereas renilla luciferase protein was V5 tagged. By Western blotting, levels of firefly luciferase were reduced in response to BRAF and MEK inhibitors, while renilla luciferase remained unaffected (Fig. 1C). Furthermore, decreased fluorescence of EGFP in response to vemurafenib and AZD6244 could be measured by Western blotting and flow cytometry. In agreement with our dual luciferase assays, no basal expression of the EGFP-firefly luciferase was detected in the absence of GAL4-ELK1 (Fig. 1D). These *in vitro* data demonstrate the generation of ERK1/2 reporter assays.

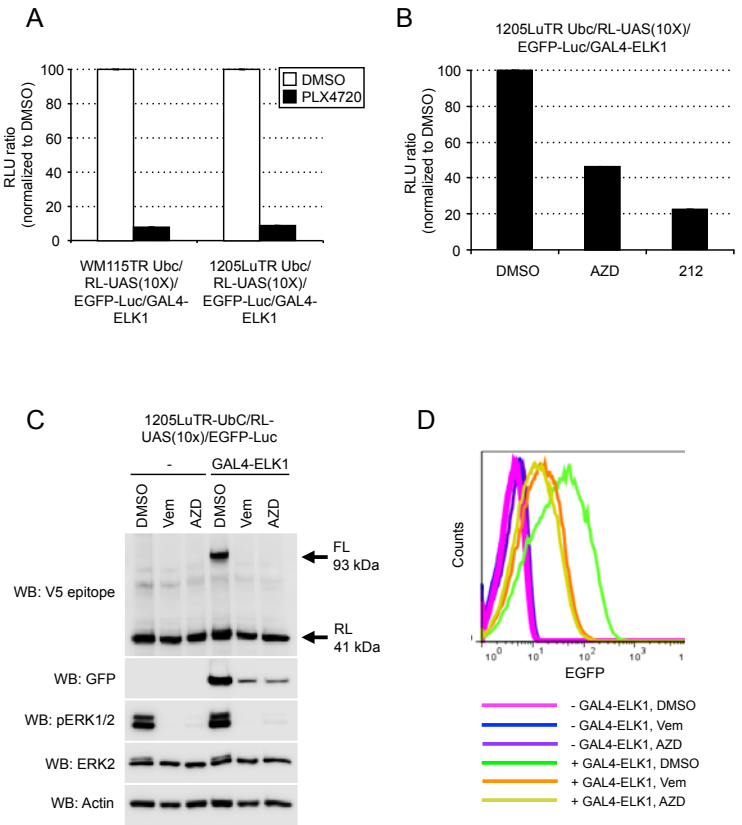

Figure 1. Use of GAL4-ELK1 reporter melanoma cells *in vitro*. **(A)** WM115TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL-ELK1 and 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 cells were treated with DMSO or PLX4720 (1μM) for 24 hours. Dual-luciferase assays were performed. Columns represent the average of three separate experiments; error bars represent standard error. (**B**) 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 cells were treated with either DMSO, AZD6244 (3.3μM) or GSK1120212 (65nM) for 24 hours. Dual-luciferase assays were performed, as above. Columns represent the average of three separate experiments; error bars represent standard error. (**C**) 1205TR Ubc/RL-UAS(10x)/EGFP-Luc (Parental) and 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 (GAL4-ELK1) cells were treated overnight with either DMSO, vemurafenib (1μM) or AZD6244 (3.3μM) and lysed. Lysates were analyzed by Western blotting, as indicated. Anti-V5 antibody allows for the detection of both EGFP-firefly luciferase (FL) and renilla luciferase (RL) at 93 kDa and 41 kDa, respectively. (**D**) 1205TR Ubc/RL-UAS(10x)/EGFP-Luc (- GAL4-ELK1) and 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 (+ GAL4-ELK1) cells were treated overnight with either DMSO, vemurafenib (1μM) or AZD6244 (3.3μM). Fluorescence of EGFP was measured by flow cytometry.

iii) Test for PLX4720 inhibition of ERK activity and re-establishment of ERK activity during relapse phase in xenograft assays *in vivo*.

In order to test the application of this system in an *in vivo* setting, xenograft models were established by injecting 1205Lu reporter cells intradermally into athymic nude mice. Cells were allowed to form tumors for 11 days. At this time point, tumor volume and renilla luciferase expression was comparable between vehicle-treated mice and PLX4720-treated mice. However, after two days of treatment, firefly luciferase expression was significantly lower in PLX4720-treated mice than in vehicle-treated mice when normalized for tumor volume (Fig. 2A). Tumor volume was determined by digital caliper measurements as opposed to renilla luciferase intensity measurements due to less variability. Despite decreased ERK1/2 signaling beginning at day 2 of treatment, noticeable differences in growth between the two treatment groups did not occur until day 7 of treatment (Fig 2B & 2C). Inhibition of ERK1/2 signaling was observed in all PLX4720-treated mice and persisted beyond 14 days of treatment. These data confirm the use of this system *in vivo*. Thus, we have fulfilled a major aim of this grant, to develop an ERK1/2 activity reporter in melanoma cell xenografts to measure PLX4720 inhibition of mutant BRAF-MEK-ERK1/2 signaling.

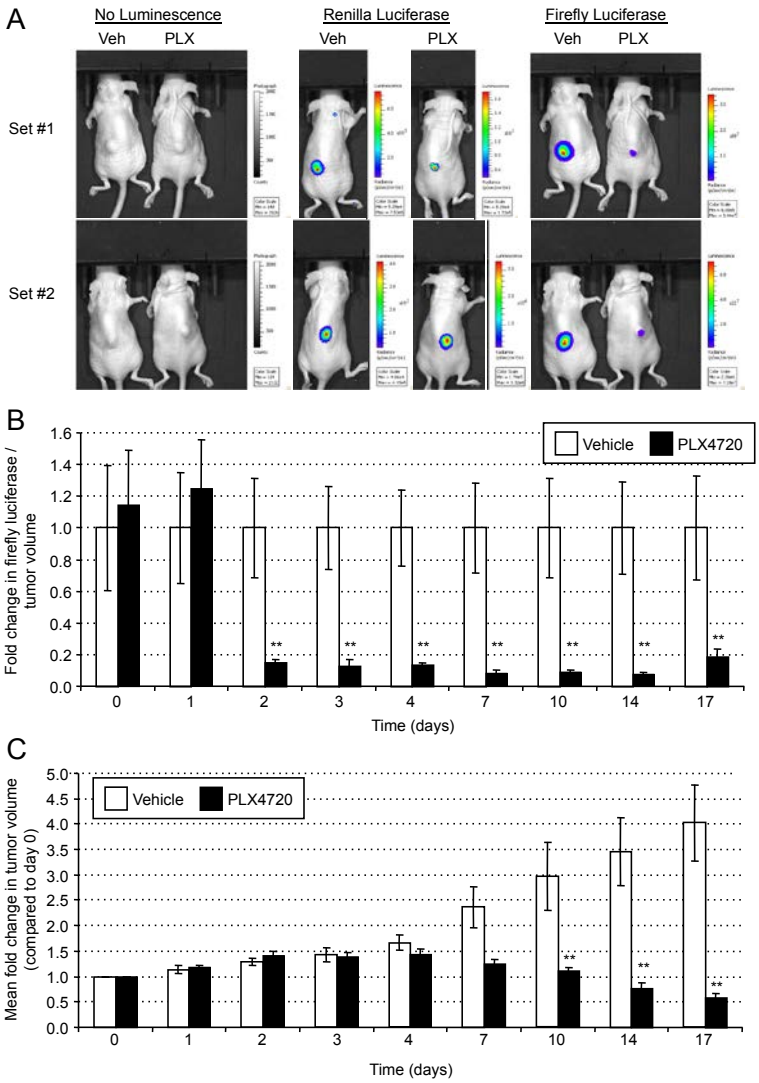

Figure 2. ERK1/2 inhibition preceded tumor suppression *in vivo* by days. **(A**) Xenograft models of 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 cells in mice that were fed either AIN-76A chow or AIN-76A with 417mg/kg PLX4720 chow were imaged for no luminescence, renilla luciferase, and firefly luciferase using a one minute exposure time. Representative images are shown from sixteen mice (eight mice per arm) from the day 3 time point. **(B)** Quantification of firefly luminescence. Columns represent fold change in firefly luciferase signal intensity adjusted for tumor volume (digital caliper measurement) compared to mean vehicle firefly luciferase signal intensity. Error bars represent standard error. **p-value<0.0001 based on unpaired, student t-test. **(C)** Mean fold change in tumor volume in 1205TR Ubc/RL-UAS(10x)/EGFP-Luc/GAL4-ELK1 xenografts in mice fed either AIN-76A chow or AIN-76A with 417mg/kg PLX4720 chow. Tumor volume was determined by digital caliper measurements as opposed to renilla luciferase intensity measurements due to less variability in the reading caused by the rapid turnover of the renilla signal. Error bars represent standard error. **p-value<0.0001 based on mixed effects model and Tukey-corrected pairwise comparisons.

Task 2. To determine the mechanism of mutant RAS-mediated resistance to PLX4032 in B-RAF^{V600E} melanomas *in vitro*.

In the second part of this grant, we examined for alterations in components of the ERK1/2 signaling pathway during acquired resistance of mutant BRAF^{V600E} melanoma cells to vemurafenib/PLX4720. Here, we treated mutant BRAF^{V600E} harboring WM793 melanoma cells with PLX4720 for four weeks at which time resistant cells grew out. PLX4720 is the tool compound for vemurafenib and elicits comparable actions to its clinical grade counterpart [10]. A sub-population of the resistant cells displayed a distinctive compact morphology. These resistant cells (termed WM793-Res NRAS) displayed comparable incorporation of EdU, a thymidine analog, in the presence and absence of PLX4720. Also, when embedded in 3-D type I collagen, no significant increase in annexin V staining was observed when treated with PLX4720. As expected, parental WM793 cells treated with PLX4720 displayed a significant reduction of EdU incorporation and increased annexin V staining. DNA sequencing of RAS-RAF pathway genes revealed a C to A mutation in NRAS, which leads to a glutamine to lysine substitution at position 61 (NRAS^{Q61K}), in the resistant cells. No NRAS^{Q61K} mutation was detected in parental cells. The mutational status of BRAF was unchanged in WM793-Res NRAS cells. Consistent with the known effect of the Q61K mutation of reducing the rate of intrinsic GTP hydrolysis in RAS, WM793-Res NRAS cells displayed increased RAS activity compared to WM793 cells in both the presence and absence of PLX4720. Furthermore, whereas PLX4720 treatment of parental WM793 cells resulted in inhibition of MEK1/2 and ERK1/2 phosphorylation throughout a 24 h time course, the phosphorylation of MEK and ERK1/2 remained elevated in WM793-Res NRAS cells. Comparable findings were observed upon analysis of serine 910 phosphorylation of focal adhesion kinase (FAK), a direct ERK1/2 phosphorylation site [11]. Together, these data indicate that mutation of NRAS is associated with MEK-ERK1/2 reactivation and acquired resistance to PLX4720.

i) Express RAS effector domain mutants in mutant B-RAF melanoma cells and test for resistance to PLX4032-mediated inhibition of ERK1/2 activity.

We tested whether NRAS^{Q61K} was sufficient to confer resistance to PLX4720 inactivation of ERK1/2. WM793, A375 (both BRAF^{V600E}) and WM115 (BRAF^{V600D}) cells were engineered to co-express either wild-type or Q61K NRAS. The concentration of 1 μM PLX4720 was utilized for these experiments since this dose was effective at inhibiting ERK1/2 signaling in parental WM793 but not resistant cells. Parental and wild-type NRAS-expressing WM793, WM115 and A375 cells showed a marked reduction in ERK1/2 phosphorylation with PLX4720, whereas ectopic NRAS^{Q61K}-expressing versions of all three lines did not show down-regulation of ERK1/2 phosphorylation. The effects of NRAS occur regardless of the retention of one wild-type BRAF allele, since A375 cells are homozygous for BRAF^{V600E}. Since various KRAS mutants confer differing responses to chemotherapies [12] and multiple Q61 substitutions occur in melanoma, we also tested whether expression of other Q61 NRAS mutants could also promote resistance to PLX4720. Expression of Q61H, Q61R and Q61L NRAS mutations in WM793 cells all resulted in resistance to PLX4720-induced ERK1/2 inactivation. Comparable results were seen in A375 cells when NRAS^{Q61H} and NRAS^{Q61R} mutations were expressed; NRAS^{Q61L} was not analyzed in this line. To rule out the possibility of adaption to constitutive expression of mutant NRAS, we generated isogenic, doxycycline-inducible lines for short-term expression of wild-type or Q61K NRAS in BRAF^{V600E} 1205Lu cells. PLX4720 inhibition of ERK1/2 was prevented by inducible expression of NRAS^{Q61K} over a range of PLX4720 concentrations and after prolonged drug treatment. Similarly, induction of NRAS^{Q61K} but not wild-type NRAS prevented ERK1/2 inactivation by vemurafenib, the clinical grade inhibitor. By contrast the MEK inhibitor, AZD6244, inhibited ERK1/2 phosphorylation in both NRAS^{Q61K} and NRAS^{WT} induced cells. In

sum, expression of mutant NRAS in $BRAF^{V600E/D}$ cells counteracts PLX4720 inhibition of the MEK-ERK1/2 pathway. These data are presented in Figure 3 of Kaplan, et al. (2012) SHOC2 and CRAF mediate ERK1/2 reactivation in mutant NRAS-mediated resistance to RAF inhibitor. **J. Biol. Chem.** 287:41797-41807.

ii) Express mutationally active SHOC-2 in mutant B-RAF melanoma cells and test for resistance to PLX4032-mediated inhibition of ERK1/2 activity.

We next tested whether wild-type and mutant $SHOC2^{S2A}$ were sufficient to confer resistance to PLX4720 inactivation of ERK1/2. Both forms of SHOC2 were expressed in WM793 un experiments similar to above; however, neither wild-type nor active SHOC2 was sufficient to confer resistance to PLX4032-mediated inhibition of ERK1/2 activity.

iii) Knockdown RAS isoforms (N-, H, & K-RAS) and SHOC-2 in mutant B-RAF cells that have acquired resistance to PLX4720, and test for requirement in ERK1/2 pathway re-activation.

To further analyze the role of NRAS, we isolated clonal cell lines that were heterozygous for the mutant NRAS allele. Two representative clones were utilized for subsequent experiments. WM793-Res NRAS clonal cells maintained high levels of phospho-ERK1/2 over a range of PLX4720 concentrations at both 1 h and 24 h treatment time points. We did observe inhibition of phospho-ERK1/2 in WM793-Res NRAS cells at high doses (30 mM) of PLX4720 after 1 h but the activation rebounded by 24 h. Next, we performed RNA interference experiments to determine the requirement for NRAS in the ability of resistant cells to by-pass the inhibitory effects of PLX4720. For these experiments, we utilized prolonged exposure to PLX4720 at the concentration (5 μM) at which the drug resistance was generated. siRNA-mediated knockdown of NRAS in WM793-Res NRAS clonal cells had only a minor effect on the basal levels of MEK and ERK1/2 phosphorylation in the absence of PLX4720. By contrast, PLX4720 treatment of NRAS knockdown WM793-Res NRAS cells effectively inhibited MEK and ERK1/2 phosphorylation. These findings show that NRAS is required for continued ERK1/2 activation in $BRAF^{V600E}$/$NRAS^{Q61K}$ co-expressing cells in the presence of PLX4720.

iv) Knockdown RAF paralogs (A-, B-, & C-RAF) in mutant B-RAF cells that have acquired resistance to PLX4720, and test for requirement in ERK1/2 pathway re-activation.

We determined the involvement of RAF isoforms in MEK-ERK1/2 signaling in WM793-Res NRAS clonal cells. Compared to the control siRNA knockdowns, BRAF depletion decreased MEK and ERK1/2 phosphorylation in the absence and presence of PLX4720 in multiple clones. By contrast, depletion of CRAF did not affect basal levels of phospho-MEK and phospho-ERK1/2 in the absence of PLX4720 but did inhibit MEK and ERK1/2 phosphorylation following PLX4720 treatment compared to control knockdowns. Together, these data indicate that WM793-Res NRAS cells primarily signal to MEK-ERK1/2 through BRAF in the absence of PLX4720 but utilize both CRAF and BRAF in the presence of PLX4720. These data are presented in Figure 6 of Kaplan, et al. (2012) SHOC2 and CRAF mediate ERK1/2 reactivation in mutant NRAS-mediated resistance to RAF inhibitor. **J. Biol. Chem.** 287:41797-41807.

Task 3. To test for the role of mutant RAS and SHOC2 in resistance to PLX4032 in $B\text{-}RAF^{V600E}$ melanomas *in vivo*.

i) Express RAS effector domain mutants in mutant B-RAF melanoma cells and test for bypass of PLX4032-mediated inhibition of growth.

We extended the analysis of ectopic expression of mutant NRAS in $BRAF^{V600E}$ melanoma cells to cell cycle progression and cell survival. First, we analyzed effects on S phase entry in inducible 1205LuTR $NRAS^{Q61K}$ cells. In the absence of RAS induction, treatment of 1205LuTR $NRAS^{Q61K}$ cells with PLX4720 significantly inhibited the incorporation of EdU compared to vehicle treatment. Induction of $NRAS^{Q61K}$ expression prevented PLX4720 inhibition of S-phase entry. To determine effects on cell survival, we utilized WM793 cells, which are susceptible to PLX4720-induced apoptosis in 3-D type I collagen [13]. Compared to expression of wild-type NRAS, WM793 cells ectopically expressing $NRAS^{Q61K}$ were more resistant to PLX4720-induced apoptosis. To determine if mutant NRAS expression alters PLX4720 inhibition of malignant properties of melanoma cells, soft agar assays were performed using A375 cells, which readily form colonies in these assays [14]. In the absence of PLX4720, both wild-type and mutant NRAS expressing A375 cells formed similar numbers of colonies. In the presence of PLX4720, only A375 cells expressing mutant NRAS formed colonies, albeit at lower numbers than without PLX4720 treatment. Collectively, these data suggest that expression of mutant NRAS promotes malignant properties of mutant $BRAF^{V600E}$ melanoma cells treated with PLX4720. These data are presented in Figure 4 of Kaplan, et al. (2012) SHOC2 and CRAF mediate ERK1/2 reactivation in mutant NRAS-mediated resistance to RAF inhibitor. **J. Biol. Chem.** 287:41797-41807.

To determine whether maintenance of the NRAS-RAF interaction was associated with resistance to PLX4720 by initially utilizing NRAS effector domain mutants, we generated doxycycline inducible $NRAS^{Q61K, T35S}$ and $NRAS^{Q61K, E37G}$ expression systems in the mutant BRAF cell line, 1205Lu. $RAS^{Q61K, T35S}$ interacts with RAF but not PI-3 kinase; whereas $RAS^{Q61K, E37G}$ interacts with PI-3 kinase but not RAF [15,16]. In the absence of exogenous NRAS expression, ERK1/2 phosphorylation was inhibited by PLX4720 in 1205LuTR $NRAS^{Q61K, T35S}$ and 1205LuTR $NRAS^{Q61K, E37G}$ cell lines. Following transgene induction, $NRAS^{Q61K, T35S}$ led to a partial block of PLX4720-induced ERK1/2 inactivation, whereas $NRAS^{Q61K, E37G}$ did not prevent PLX4720 inhibition of phospho-ERK1/2. Similarly, constitutive expression of $NRAS^{Q61K, T35S}$ but not $NRAS^{Q61K, E37G}$ in WM793 prevented PLX4720 inhibition of phospho-ERK1/2. In this system, $NRAS^{Q61K, T35S}$ completely prevented the inhibitory effects of PLX4720. These data suggest that $NRAS^{Q61K}$-mediated resistance to PLX4720 is associated with the RAF binding site. To test if association with RAF is required for mutant N-RAS to promote S-phase entry in the presence of PLX4720, EdU incorporation assays were performed on 1205LuTR cells expressing $NRAS^{Q61K}$ effector domain mutations. In the presence of PLX4720, $NRAS^{Q61K, T35S}$ promoted S-phase progression, whereas $NRAS^{Q61K, E37G}$ was unable to promote S-phase progression in the presence of PLX4720. We also examined resistance to apoptosis in the presence of PLX4720 using WM793 cells embedded in 3-D type-I collagen. WM793 $NRAS^{Q61K, E37G}$-expressing cells were susceptible to PLX4720-induced increases in annexin V staining, whereas $N\text{-}RAS^{Q61K, T35S}$-expressing cells displayed only a slight increase in annexin V in the presence of PLX4720. These data are presented in Figure 5 of Kaplan, et al. (2012) SHOC2 and CRAF mediate ERK1/2 reactivation in mutant NRAS-mediated resistance to RAF inhibitor. **J. Biol. Chem.** 287:41797-41807.

ii) Express mutationally active SHOC-2 in mutant B-RAF melanoma cells and test for resistance to PLX4032-mediated inhibition of growth.

Based on our findings that active SHOC2 was not sufficient to recover ERK1/2 activity in the presence of PLX4720, we did not perform growth experiments.

iii) Analysis of RAS and SHOC-2 mutations in pre-treatment, during treatment and post-release tumor samples from the PLX4032 clinical trials.

During the course of our studies both Nazarian et al [17] and Poulikakos et al [5] published that NRAS mutations are associated with acquired resistance to vemurafenib/PLX4032.

Key Research Accomplishments

1. We have established an *in vivo* ERK1/2 reporter system and utilized it to provide quantitative analysis of RAF and MEK inhibitors in tumor cells.

2. We have shown that NRAS mutations re-activate the ERK1/2 pathway and confer resistant to RAF inhibitors in mutant BRAF cells.

3. We have demonstrated that expression of mutant NRAS in mutant BRAF melanoma cells alter RAF isoform usage and scaffold molecule requirements.

Reportable Outcomes

1. Kaplan, F.M., Kugel, C.H., Dadpey, N., Shao, Y., Abel, E.V., and **Aplin, A.E.** (2012) SHOC2 and CRAF mediate ERK1/2 reactivation in mutant NRAS-mediated resistance to RAF inhibitor. **J. Biol. Chem.** 287:41797-41807.

Conclusion

It is essential to quantitatively and temporally monitor the effect of kinase inhibitors on their target pathways. We have developed a system to quantify changes in ERK1/2 signaling in tumor cells with elevated ERK1/2 activity *in vitro* and *in vivo*. We validated this system *in vitro* using mutant BRAF harboring melanoma cells and the BRAF inhibitors (PLX4720, vemurafenib) or MEK1/2 inhibitors (AZD6244, GSK1120212). Importantly, the effects of BRAF inhibition could be visualized *in vivo*, with PLX4720 greatly reducing firefly luciferase activity in melanoma xenografts. As ERK1/2 reactivation is one of the main causes of relapse of patients treated with BRAF [5,17,18], this model may serve as a means of tracking this event *in vivo*. With an improved understanding of ERK1/2 reactivation kinetics, experiments can be designed to test the ability of other clinical/preclinical reagents to block ERK1/2 reactivation in concert with BRAF inhibitors.

In the second aim, we describe melanoma cells with acquired resistance to a clinically relevant RAF inhibitor that is associated with a mutation in NRAS. We show that mutant NRAS is sufficient to confer resistance to RAF inhibitors. Furthermore, we provide mechanistic evidence that these mutant NRAS resistant cells alter their signaling connections in response to RAF inhibitor resulting to a shift in the dependency to CRAF and the RAS-RAF scaffold molecule, SHOC2.

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Appendices

N/A

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SHOC2 and CRAF Mediate ERK1/2 Reactivation in Mutant NRAS-mediated Resistance to RAF Inhibitor*[S]

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Background: Reactivation of ERK1/2 frequently underlies acquired resistance to RAF inhibitors.
Results: NRAS mutations are acquired during resistance to RAF inhibitors and promote CRAF and SHOC2-modulated ERK1/2 pathway re-activation.
Conclusion: NRAS mutations in mutant BRAF cells alter RAF isoform and SHOC2 usage in the presence of RAF inhibitor.
Significance: These studies delineate mechanisms mediating RAF inhibitor resistance in mutant BRAF cells.

ERK1/2 signaling is frequently dysregulated in tumors through BRAF mutation. Targeting mutant BRAF with vemurafenib frequently elicits therapeutic responses; however, durable effects are often limited by ERK1/2 pathway reactivation via poorly defined mechanisms. We generated mutant $BRAF^{V600E}$ melanoma cells that exhibit resistance to PLX4720, the tool compound for vemurafenib, that co-expressed mutant (Q61K) NRAS. In these $BRAF^{V600E}$/$NRAS^{Q61K}$ co-expressing cells, re-activation of the ERK1/2 pathway during PLX4720 treatment was dependent on NRAS. Expression of mutant NRAS in parental $BRAF^{V600}$ cells was sufficient to by-pass PLX4720 effects on ERK1/2 signaling, entry into S phase and susceptibility to apoptosis in a manner dependent on the RAF binding site in NRAS. ERK1/2 activation in $BRAF^{V600E}$/$NRAS^{Q61K}$ cells required CRAF only in the presence of PLX4720, indicating a switch in RAF isoform requirement. Both ERK1/2 activation and resistance to apoptosis of $BRAF^{V600E}$/$NRAS^{Q61K}$ cells in the presence of PLX4720 was modulated by SHOC-2/Sur-8 expression, a RAS-RAF scaffold protein. These data show that NRAS mutations confer resistance to RAF inhibitors in mutant BRAF cells and alter RAF isoform and scaffold molecule requirements to re-activate the ERK1/2 pathway.

RAS activation of the extracellular signal-regulated kinase (ERK)[3]/mitogen-activated protein (MAP) kinase cascade plays a critical role in the proliferation and survival of normal and malignant cells (1–3). In this pathway, GTP-loading of RAS isoforms (HRAS, NRAS, KRAS) leads to the membrane recruitment of RAF serine-threonine kinases (ARAF, BRAF, CRAF). RAS binds RAF and facilitates RAF activation and initiation of a kinase cascade that signals to the dual specificity kinases, MAPK/ERK kinases 1 and 2 (MEK1/2), and subsequently to ERKs 1 and 2. The activity at each level of the cascade is fine tuned by additional layers of control. Substitute MAP3Ks, such as Cot1/TPL2, can activate MEKs in certain conditions (4). Splice variants have been identified for pathway components including BRAF (5), MEK (6), and ERK1 (7), which display altered activity and/or specificity. Negative feedback pathways including ERK1/2-dependent up-regulation of sprouty proteins and dual-specificity phosphatases serve to dampen pathway output at various levels (8–11). Additionally, scaffold molecules interact with pathway components and co-ordinate their activation at distinct subcellular locales. Among these scaffolds is Soc-2 (suppressor of clear) homolog, SHOC2/Sur8. This leucine-rich repeat protein was originally identified in *Caenorhabditis elegans*-based screens for regulators of growth factor receptor-RAS signaling (12, 13). In mammalian cells, SHOC2 promotes RAS-RAF association and positively enhances signaling through the ERK1/2 pathway (14).

Signaling downstream of mutant BRAF represents one context in which it is critical to understand the complex interactions in the ERK1/2 pathway. Approximately, 8% of human tumors harbor a BRAF mutation, the majority of which are phosphomimetic V600E substitutions within the activation loop of BRAF (15). High frequencies of BRAF mutations have been identified in melanomas, thyroid carcinomas, and colorectal tumors (15). $BRAF^{V600E}$ signals as a monomer and in a RAS-independent manner to constitutively activate MEK (16). Small molecule inhibitors, such as vemurafenib (PLX4032) and its tool analog, PLX4720, have been developed. Despite being pan RAF inhibitors *in vitro*, vemurafenib selectively inhibits $BRAF^{V600E}$ signaling in cells (17–19). The majority of late stage, mutant BRAF melanoma patients treated with vemurafenib display short-term tumor shrinkage (18, 20, 21). Furthermore, vemurafenib gives improved overall survival and progression-free survival compared with standard chemotherapy (22). Unfortunately, the long-term effects of vemurafenib are frequently hampered by resistance mechanisms (23–25).

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[S] This article contains supplemental Figs. S1 and S2.
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[3] The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated kinase; MEK1/2, MAPK/ERK kinases 1 and 2; EdU, 5-ethynyl-2′-deoxyuridine; FAK, focal adhesion kinase.

While multiple modes of resistance to vemurafenib are likely to occur, one over-arching mechanism involves re-activation of the ERK1/2 pathway (25). Mutations in NRAS and BRAF are usually mutually exclusive; however, attention has focused on mutations in NRAS in the resistance to vemurafenib. This is largely based on the paradoxical effects of RAF inhibitors in cells with mutant or activated RAS (19, 26–28). Mutations in NRAS were recently identified in two tumor re-growths in one mutant BRAF melanoma patient (23) and four out of nineteen patients (29) following progression on vemurafenib. In the former study, $NRAS^{Q61R}$ and $NRAS^{Q61K}$ mutations were associated with ERK1/2 pathway re-activation (23). Despite this knowledge, the mechanism whereby mutant NRAS prevents vemurafenib from inhibiting MEK-ERK1/2 signaling in cells expressing mutant BRAF remains unknown. Here, we isolated mutant BRAF melanoma cell lines with secondary resistance to PLX4720 (the tool compound for vemurafenib). A subset of the resistant cells co-expressed $NRAS^{Q61K}$ and $BRAF^{V600E}$. In parental cells, expression of $NRAS^{Q61K}$ was sufficient to provide resistance to PLX4720 in a manner dependent on the RAF binding domain. ERK1/2 reactivation in resistant cells was dependent on NRAS and regulated by both BRAF and CRAF. Furthermore, ERK1/2 reactivation in the presence of PLX4720 was reduced following depletion of the RAS-RAF scaffold molecule, SHOC2. SHOC2 was required, at least partially, for the survival of $BRAF^{V600E}$/$NRAS^{Q61K}$ cells in the presence of PLX4720. These data provide novel insight into a mechanism of ERK1/2 pathway re-activation in RAF inhibitor-resistant cells.

EXPERIMENTAL PROCEDURES

Inhibitors—PLX4720 was kindly provided by Dr. Gideon Bollag and Plexxikon Inc. (Berkeley, CA). AZD6244 was purchased from Selleck Chemicals LLC (Houston, TX).

Cloning and Stable Cell Line Generation—Wild-type and mutant NRAS were cloned from cDNA using the following primers: forward CACCATGACTGAGTACAAACTGGTGGTG and reverse TTACATCACCACACATGGCAATCCC. $NRAS^{Q61K,\ T35S}$ and $NRAS^{Q61K,\ E37G}$ were constructed following the QuikChange protocol (Stratagene, La Jolla, CA). $NRAS^{Q61K}$ was the template for the following primers: T35S forward GTAGATGAATATGATCCCTCCATAGAGGATTCTTACAGA and T35S reverse TCTGTAAGAATCCTCTATGGAGGGATCATATTCATCTAC and E37G forward GAATATGATCCCACCATAGGGGATTCTTACAGAAACAA and E37 reverse TTGTTTTCTGTAAGAATCCCCATGGTGGGATCATATTC. All DNA constructs were sequence verified. Lentiviral particles and stable cell lines were made as previously described (30). Transgene expression was induced with 0.1 μg/ml doxycycline.

Cell Culture—Melanoma cells were cultured, as previously described (31). WM793, WM115, WM1366, Sbcl2, and 1205Lu were cultured in MCDB 153 containing 20% Leibovitz L-15 medium, 2% FBS, 5 μg/ml insulin and penicillin/streptomycin. A375 cells were cultured in DMEM with 10% FBS.

Generation of Resistant Cell Lines—WM793 cells were cultured in the aforementioned MCDB 153 medium in the presence of 5 μM PLX4720 for 4 weeks. Medium containing PLX4720 was replenished every 2 days. Resistant clonal lines were generated by seeding cells at low density and allowing isolated colonies to form. These colonies were picked, and cells expanded in the continued presence of PLX4720.

siRNA Transfections—WM793 cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, MA). Non-targeting control, B-RAF #1, and C-RAF sequences were as previously described (26). NRAS (CACCAUAGAGGAUUCUUAC) and SHOC2 (#1: GACCUUAGCUAGAAAUUGC; #2: GAAAUUGGUACACUGGAGA; #3: GAGGUAGUAUAGUUAGAUA) siRNAs were purchased from Dharmacon (Lafayette, CO). Cells were transfected for 72 h with a final siRNA concentration of 25 nM before subsequent treatment or analysis.

Western Blotting—Western blotting was performed, as previously described (26). Immunoreactivity was detected using peroxidase-conjugated secondary antibodies and chemiluminescence substrate (Pierce). Chemiluminescence was detected using a Versadoc Imaging system (Bio-Rad). The following antibodies were purchased from Cell Signaling Technology, (Beverley, MA): phospho-ERK1/2 (Thr-202/Tyr-204, #4377), MEK1 (#9124), phospho-MEK1 (#9121), phospho-Akt (Thr-308, #2965) phospho-Akt (Ser-473, #6942) total Akt (#2965), and total FAK (BD Biosciences, San Jose, CA). B-RAF (sc-5284), C-RAF/RAF-1 (sc-133), ERK1/2 (sc-094), ERK2 (sc-1647), and NRAS (sc-31) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Shoc2/Sur8 (ab32982) antibody was purchased from Abcam (Cambridge, MA). Phospho-FAK-S910 was purchased from BIOSOURCE Int. (Camarillo, CA).

Three-dimensional (3-D) Collagen Gels and Apoptosis Assays—Collagen gels were cast by mixing the following on ice: Eagle's Minimum Essential Medium (Lonza, Inc. Walkersville, MD), 2 mM L-glutamine, 2% FBS, 0.15% sodium bicarbonate, and 0.8 mg/ml bovine type-I collagen (Organogenesis Inc., Canton, MA). Cells were seeded in 2 ml collagen gels and incubated at 37 °C for 30 min. After polymerization, the collagen gel lattice was overlaid with 2 ml of medium at 37 °C for 48 h. 3-D collagen gels were dissolved in 1 mg/ml collagenase (Sigma-Aldrich) solution to release cells. Cells were resuspended in 100 μl of binding buffer (10 mM HEPES, 0.14 M sodium chloride, 2.5 mM calcium chloride), stained with 5 μl of Annexin V-APC (BD Biosciences) for 15 min and finally an additional 400 μl of binding buffer was added. Apoptosis was analyzed by flow cytometry on the FACSCalibur (BD Biosciences). Data were analyzed using Flowjo software (Three Star, Inc. Ashland, OR).

EdU (5-Ethynyl-2′-deoxyuridine) Incorporation Assays—Parental WM793 cells and WM793-Res NRAS cells were cultured overnight in the absence of PLX4720. Cells were treated with DMSO or PLX4720 for 48 h before the addition of 10 μM EdU for another 16 h. Cells were then processed using the Click-iT™ EdU Alexa Fluor 647 Flow Cytometry Assay kit (Invitrogen, Carlsbad, CA) for flow cytometry analysis.

Soft Agar Assays—Cells (3×10^3 cells/ml) were grown in 0.3% soft agar, as previously described (30). Cells were grown for 14 days, replacing the medium every 3 days. Five random fields per chamber were acquired using NIS-Elements software from Nikon.

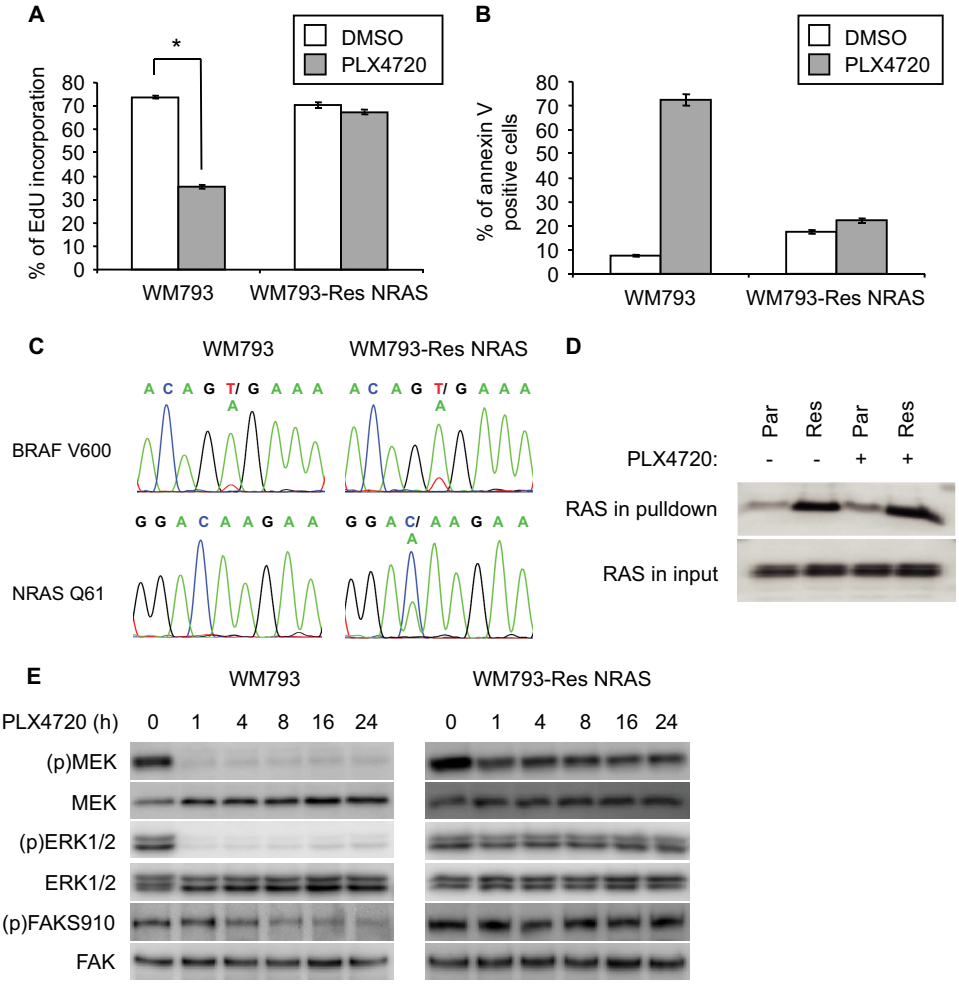

FIGURE 1. **Mutation in NRAS associated with acquired resistance to RAF inhibitor in mutant BRAF cells.** *A*, WM793 and WM793-Res NRAS cells were cultured overnight in the absence of PLX4720 and then treated with DMSO and 1 μM PLX4720 for 48 h. Finally, EdU incorporation was performed for 16 h in the presence/absence of PLX4720. Assays were performed in triplicate. Error bars, S.D. *, $p < 0.05$. *B*, WM793 and WM793-Res cells were cultured overnight in the absence of PLX4720, embedded in 3-D collagen and then treated with either DMSO or 1 μM PLX4720 for 48 h. Apoptosis was quantitated using annexin V staining. Assays were performed in triplicate. Error bars, S.D. *C*, sequencing of WM793-Res pooled cells for BRAF and NRAS. *D*, WM793 parental and WM793-Res NRAS cells were incubated in the absence of PLX4720 overnight and then in the absence/presence of 5 μM PLX4720 for an additional 24 h. RAS pull-down assays using GST-RAF-RBD (RAS binding domain) were performed. Shown are Western blot data for NRAS from the input and pulldown samples. *E*, WM793 and WM793-Res NRAS cells were seeded overnight in the absence of PLX4720 and then treated with 1 μM PLX4720 for times ranging from 0 to 24 h. Samples were analyzed by Western blotting for phospho-MEK, total MEK, phospho-ERK1/2, total ERK1/2, phospho-S910 FAK, and total FAK.

Statistical Analysis—Statistical analysis of the data was performed using an unpaired Student's *t* test assuming unequal variance.

RESULTS

Acquired Resistance of Mutant BRAF^{V600E} Melanoma Cells to PLX4720 Is Associated with Mutational Activation of NRAS—To examine acquisition of resistance to RAF inhibitors, we treated mutant BRAF^{V600E} harboring WM793 melanoma cells with PLX4720 for 4 weeks at which time resistant cells grew out. PLX4720 is the tool compound for vemurafenib and elicits comparable actions to its clinical grade counterpart (19). A subpopulation of the resistant cells displayed a distinctive compact morphology. These resistant cells (termed WM793-Res NRAS, *vide infra*) displayed comparable incorporation of EdU, a thymidine analog, in the presence and absence of PLX4720 (Fig. 1*A*). Also, when embedded in 3-D type I collagen, no significant increase in annexin V staining was observed when treated with PLX4720 (Fig. 1*B*). As expected, parental WM793 cells treated with PLX4720 displayed a significant reduction of EdU incorporation and increased annexin V staining (Fig. 1, *A* and *B*). DNA sequencing of RAS-RAF pathway genes revealed a C to A mutation in NRAS, which leads to a glutamine to lysine substitution at position 61 (NRAS^{Q61K}), in the resistant cells (Fig. 1*C*). No NRAS^{Q61K} mutation was detected in parental cells. The mutational status of BRAF was unchanged in WM793-Res NRAS cells (Fig. 1*C*). Consistent with the known effect of the Q61K mutation of reducing the rate of intrinsic GTP hydrolysis in RAS, WM793-Res NRAS cells displayed increased RAS activity compared with WM793 cells in both the presence and absence of PLX4720 (Fig. 1*D*). Furthermore, whereas PLX4720 treatment of parental WM793 cells resulted in inhibition of MEK1/2 and ERK1/2 phosphorylation throughout a 24 h time course, the phosphorylation of MEK and ERK1/2 remained elevated in WM793-Res NRAS cells (Fig. 1*E*). Comparable findings were observed upon analysis of serine 910 phosphorylation

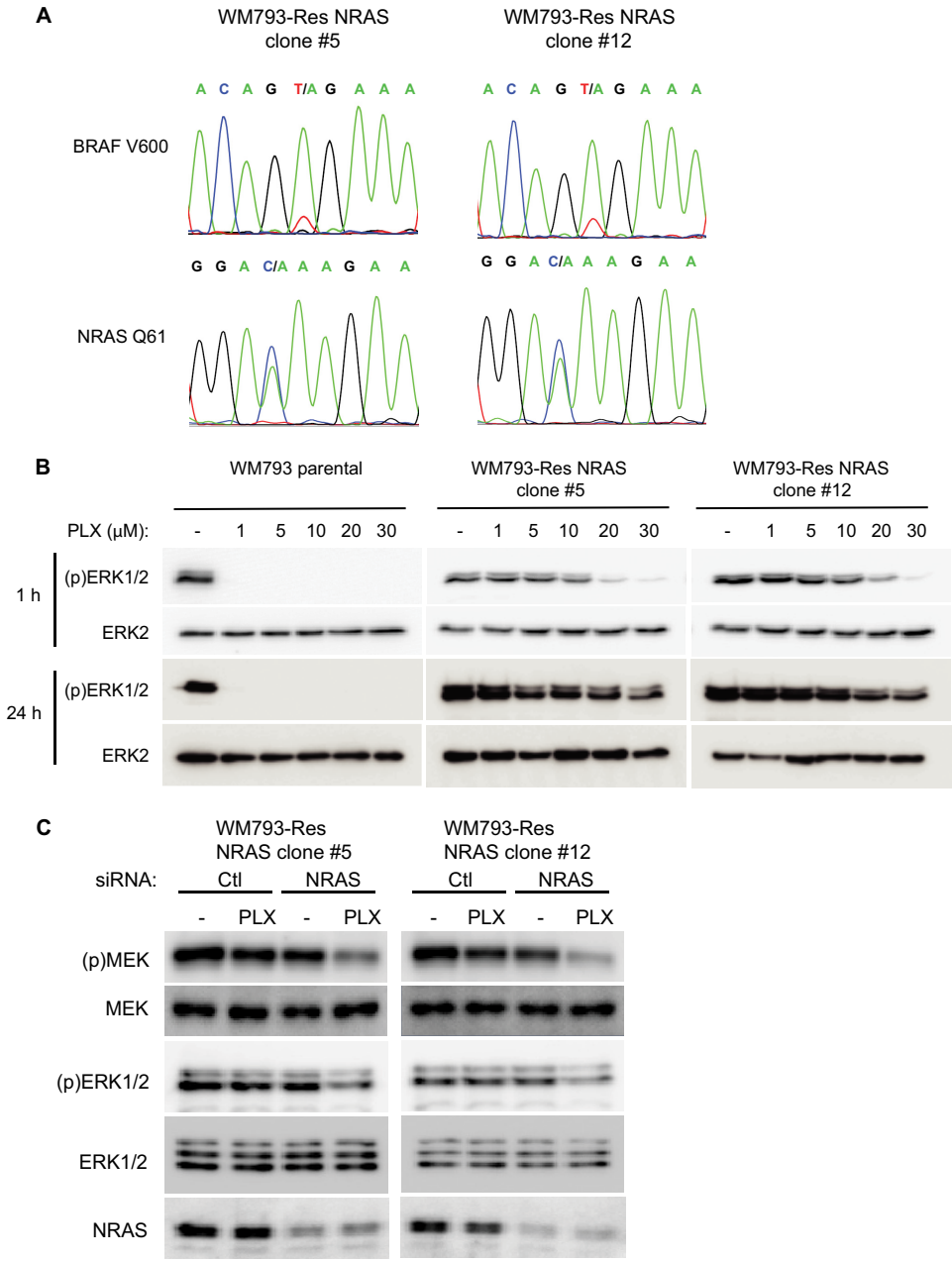

FIGURE 2. **Activation of MEK-ERK1/2 in resistant clones is dependent on NRAS in the presence of RAF inhibitor.** *A*, sequencing of WM793-Res NRAS clones #5 and #12 for BRAF exon 15 and NRAS exon 2. *B*, Parental WM793, WM793Res NRAS clone #5, and WM793Res NRAS clone #12 were seeded overnight in the absence of PLX4720 and then treated with 0, 1, 5, 10, 20, 30 μM PLX4720 for 1 h or 24 h, as indicated. Cell lysates were analyzed by Western blotting for phospho-ERK1/2 and total ERK2. *C*, WM793-Res NRAS cells (clones #5 and #12) were transfected with control (*Ctl*) or NRAS siRNA. Post-transfection, cells were treated with either DMSO (−) or 5 μM PLX4720 for 72 h. Inhibitor was replenished after 48 h. Lysates were analyzed by Western blotting for phospho-MEK, total MEK, phospho-ERK1/2, total ERK1/2, and NRAS.

of focal adhesion kinase (FAK), a direct ERK1/2 phosphorylation site (32). Together, these data indicate that mutation of NRAS is associated with MEK-ERK1/2 reactivation and acquired resistance to PLX4720.

NRAS Is Required for Resistance to PLX4720—To further analyze the role of NRAS, we isolated clonal cell lines that were heterozygous for the mutant NRAS allele (Fig. 2*A*). Out of 13 clones isolated, 8 (61.5%) harbored NRAS mutations and 5 (38.5%) were wild-type for NRAS. Two representative clones (5 and 12) were utilized for subsequent experiments. WM793-Res NRAS clonal cells maintained high levels of phospho-ERK1/2 over a range of PLX4720 concentrations at both 1 and 24 h treatment time points (Fig. 2*B*). We did observe inhibition of phospho-ERK1/2 in WM793-Res NRAS cells at high doses (30 μM) of PLX4720 after 1 h but the activation rebounded by 24 h.

Next, we performed RNA interference experiments to determine the requirement for NRAS in the ability of resistant cells to by-pass the inhibitory effects of PLX4720. For these experiments, we utilized prolonged exposure to PLX4720 at the concentration (5 μM) at which the drug resistance was generated. siRNA-mediated knockdown of NRAS in WM793-Res NRAS#5 and #12 cells had only a minor effect on the basal levels

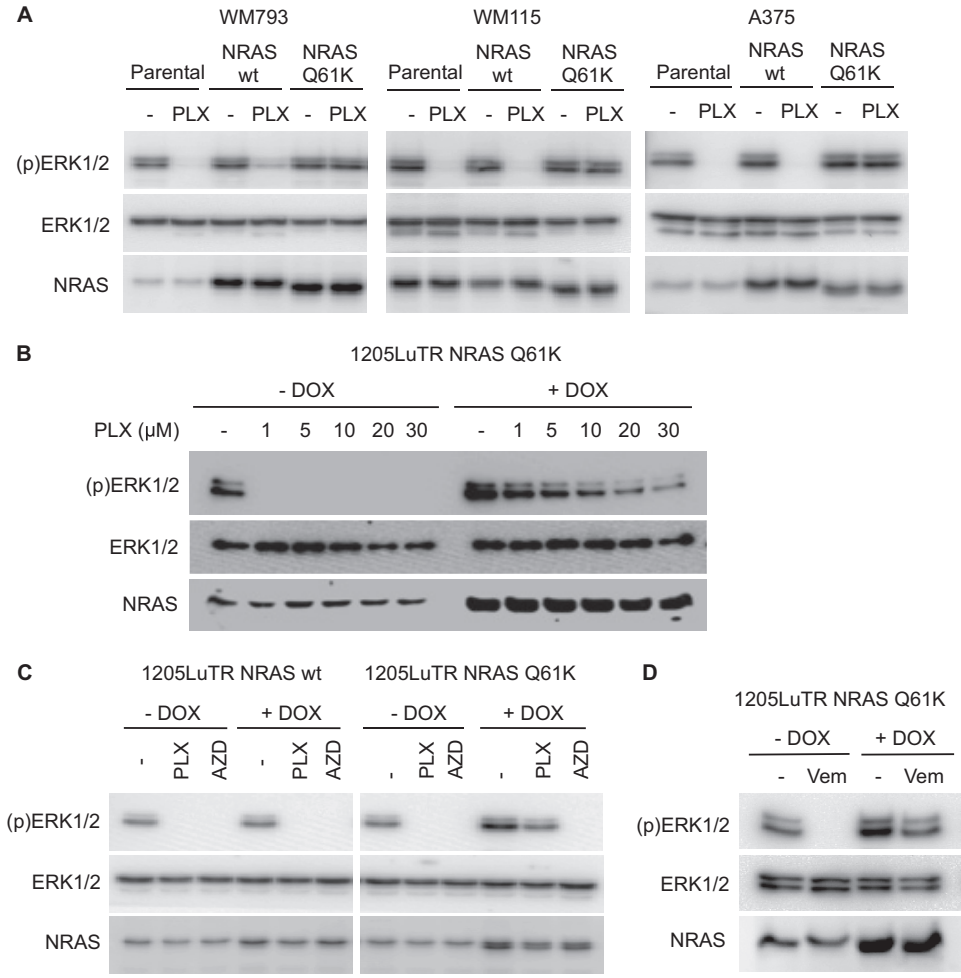

FIGURE 3. **NRAS^{Q61K} expression is sufficient for resistance to PLX4720/vemurafenib inhibition of ERK1/2.** *A*, WM793, WM115, and A375 parental cells and lines constitutively expressing NRAS wild-type (wt) and NRAS^{Q61K} were treated with DMSO or 1 μM PLX4720 for 1 h. Western blots were performed for phospho and total ERK1/2. *B*, 1205LuTR-Lu NRAS^{Q61K} cells were cultured with 100 ng/ml doxycycline (+DOX) for 16 h to induce ectopic NRAS expression and then treated with DMSO or a range of concentrations of PLX4720 for 1 h. Western blot analysis was performed on the lysates with the indicated antibodies. *C*, inducible 1205LuTR-Lu NRAS wild-type and NRAS^{Q61K} cells were treated −/+ DOX for 16 h and then with DMSO, 5 μM PLX4720 or 3.3 μM AZD for 16 h. Lysates were subjected to Western blot analysis. *D*, inducible 1205LuTR-Lu NRAS^{Q61K} cells were treated −/+ DOX for 16 h to induce NRAS expression. Cells were then treated with DMSO or 1 μM vemurafenib for 1 h and subjected to Western blot analysis.

of MEK and ERK1/2 phosphorylation in the absence of PLX4720 (Fig. 2*C*). By contrast, PLX4720 treatment of NRAS knockdown WM793-Res NRAS cells effectively inhibited MEK and ERK1/2 phosphorylation (Fig. 2*C*). These findings show that NRAS is required for continued ERK1/2 activation in BRAF^{V600E}/NRAS^{Q61K} co-expressing cells in the presence of PLX4720.

Mutant NRAS Is Sufficient Prevent PLX4720 Inhibition of the ERK1/2 Pathway in Mutant BRAF Cells—We next tested whether NRAS^{Q61K} was sufficient to confer resistance to PLX4720 inactivation of ERK1/2. WM793, A375 (both BRAF^{V600E}), and WM115 (BRAF^{V600D}) cells were engineered to co-express either wild-type or Q61K NRAS. The concentration of 1 μM PLX4720 was utilized for these experiments since this dose was effective at inhibiting ERK1/2 signaling in parental WM793 but not resistant cells (Fig. 2*B*). Parental and wild-type NRAS-expressing WM793, WM115 and A375 cells showed a marked reduction in ERK1/2 phosphorylation with PLX4720, whereas ectopic NRAS^{Q61K}-expressing versions of all three lines did not show down-regulation of ERK1/2 phosphorylation (Fig. 3*A*). The effects of NRAS occur regardless of the retention of one wild-type BRAF allele, since A375 cells are homozygous for BRAF^{V600E} (supplemental Fig. S1*A*). Because various KRAS mutants confer differing responses to chemotherapies (33) and multiple Q61 substitutions occur in melanoma, we also tested whether expression of other Q61 NRAS mutants could also promote resistance to PLX4720. Expression of Q61H, Q61R, and Q61L NRAS mutations in WM793 cells all resulted in resistance to PLX4720-induced ERK1/2 inactivation (supplemental Fig. S1*B*). Comparable results were seen in A375 cells when NRAS^{Q61H} and NRAS^{Q61R} mutations were expressed (supplemental Fig. S1*C*); NRAS^{Q61L} was not analyzed in this line.

To rule out the possibility of adaption to constitutive expression of mutant NRAS, we generated isogenic, doxycycline-inducible lines for short-term expression of wild-type or Q61K NRAS in BRAF^{V600E} 1205Lu cells. PLX4720 inhibition of ERK1/2 was prevented by inducible expression of NRAS^{Q61K} over a range of PLX4720 concentrations (Fig. 3*B*) and after prolonged drug treatment (Fig. 3*C*). Similarly, induction of NRAS^{Q61K} but not wild-type NRAS prevented ERK1/2 inacti-

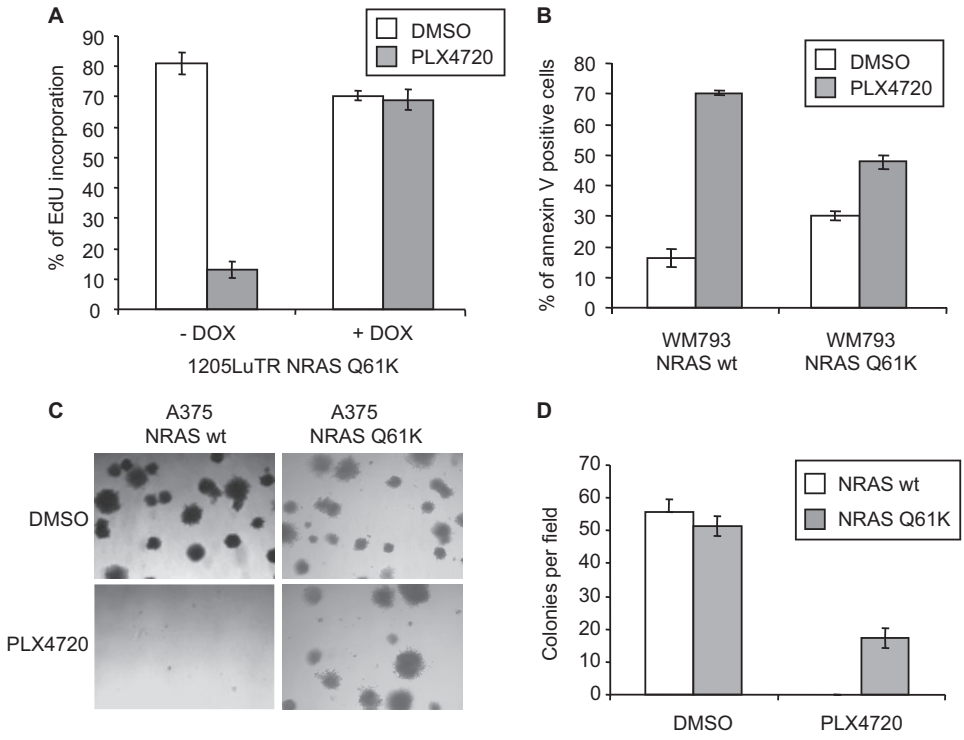

FIGURE 4. **Expression of NRAS^{Q61K} promotes proliferation and inhibits apoptosis in the presence of PLX4720.** *A*, 1205Lu-TR NRAS^{Q61K} cells were cultured + DOX (100 ng/ml) for 16 h. Cells were then treated with 1 μM PLX4720 or DMSO for 48 h. EdU was added and analysis was performed after 16 h. Assays were performed in triplicate. Error bars, standard deviation. *B*, WM793 and WM115 cells expressing NRAS wild-type and NRAS^{Q61K} cells were embedded into 3-D collagen and treated with either DMSO or 1 μM PLX4720 for 48 h. Assays were performed in triplicate and apoptosis was quantitated using annexin V staining. Error bars, S.D. *C*, A375 cells expressing wild-type NRAS or NRAS^{Q61K} were embedded into soft agar and treated with either DMSO or 1 μM PLX4720. Representative pictures were taken after 2 weeks. *D*, five independent fields were used to determine the average number of colonies per field. Error bars, S.D.

vation by vemurafenib, the clinical grade inhibitor (Fig. 3*D*). By contrast the MEK inhibitor, AZD6244, inhibited ERK1/2 phosphorylation in both NRAS^{Q61K}- and NRAS^{WT}-induced cells (Fig. 3*C*). In summary, expression of mutant NRAS in BRAF$^{V600E/D}$ cells counteracts PLX4720 inhibition of the MEK-ERK1/2 pathway.

Mutant NRAS Prevents PLX4720 Inhibition of S Phase Entry and Induction of Apoptosis in Mutant BRAF Cells—We extended the analysis of ectopic expression of mutant NRAS in BRAF^{V600E} melanoma cells to cell cycle progression and cell survival. First, we analyzed effects on S phase entry in inducible 1205LuTR NRAS^{Q61K} cells. In the absence of RAS induction, treatment of 1205LuTR NRAS^{Q61K} cells with PLX4720 significantly inhibited the incorporation of EdU compared with vehicle treatment (Fig. 4*A*). Induction of NRAS^{Q61K} expression prevented PLX4720 inhibition of S-phase entry (Fig. 4*A*). To determine effects on cell survival, we utilized WM793 cells, which are susceptible to PLX4720-induced apoptosis in 3-D type I collagen (34). Compared with expression of wild-type NRAS, WM793 cells ectopically expressing NRAS^{Q61K} were more resistant to PLX4720-induced apoptosis (Fig. 4*B*). To determine if mutant NRAS expression alters PLX4720 inhibition of malignant properties of melanoma cells, soft agar assays were performed using A375 cells, which readily form colonies in these assays (30). In the absence of PLX4720, both wild-type and mutant NRAS expressing A375 cells formed similar numbers of colonies (Fig. 4*C* and quantitated in 4*D*). In the presence of PLX4720, only A375 cells expressing mutant NRAS formed colonies, albeit at lower numbers than without PLX4720 treatment (Fig. 4, *C* and *D*). Collectively, these data suggest that expression of mutant NRAS promotes malignant properties of mutant BRAF^{V600E} melanoma cells treated with PLX4720.

NRAS-RAF Association Correlates with Resistance to PLX4720—We next determined whether maintenance of the NRAS-RAF interaction was associated with resistance to PLX4720 by initially utilizing NRAS effector domain mutants. We generated doxycycline inducible NRAS^{Q61K,T35S} and NRAS$^{Q61K,\ E37G}$ expression systems in the mutant BRAF cell line, 1205Lu. RAS^{Q61K,T35S} interacts with RAF but not PI-3 kinase or Ral-GDS; whereas RAS^{Q61K,E37G} interacts with PI-3 kinase and Ral-GDS but not RAF (14, 35). In the absence of exogenous NRAS expression, ERK1/2 phosphorylation was inhibited by PLX4720 in 1205LuTR NRAS^{Q61K,T35S} and 1205LuTR NRAS^{Q61K,E37G} cell lines (Fig. 5*A*). Following transgene induction, NRAS^{Q61K,T35S} led to a partial block of PLX4720-induced ERK1/2 inactivation, whereas NRAS^{Q61K,E37G} did not prevent PLX4720 inhibition of phospho-ERK1/2 (Fig. 5*A*). NRAS^{Q61K,E37G} induction promoted Akt activity demonstrating biological activity of this effector domain mutant in this system (Fig. 5*A*) Similarly, constitutive expression of NRAS^{Q61K,T35S} but not NRAS^{Q61K,E37G} in WM793 prevented PLX4720 inhibition of phospho-ERK1/2 (Fig. 5*B*). In this system, NRAS^{Q61K,T35S} completely prevented the inhibitory effects of PLX4720. These data suggest that NRAS^{Q61K}-mediated resistance to PLX4720 is associated with the RAF binding site.

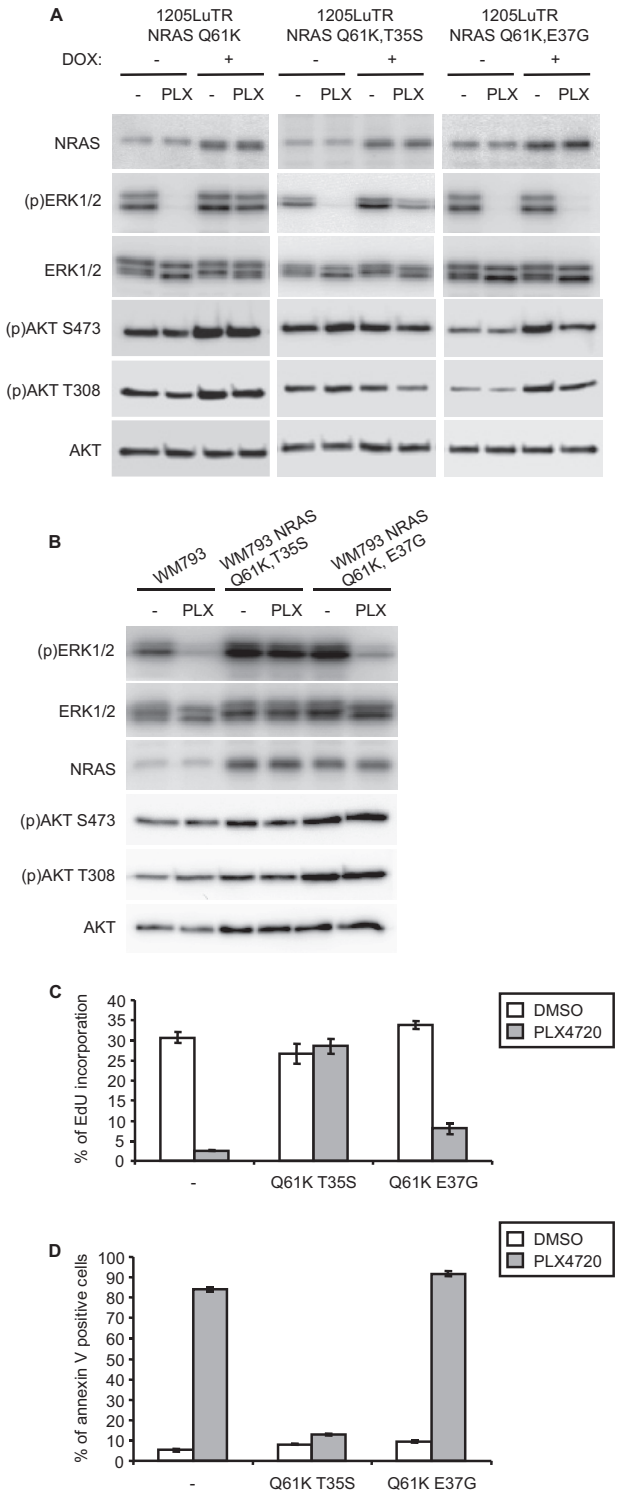

FIGURE 5. **NRAS^{Q61K}-mediated resistance is associated with RAF binding.** *A*, 1205LuTR-Lu NRAS^{Q61K}, NRAS^{Q61K,T35S}, and NRAS^{Q61K,E37G} cells were cultured +DOX for 16 h to induce NRAS expression. Cells were then treated with 1 μM PLX4720 or DMSO (−) for 1 h and cell lysates subjected to Western blot analysis for the indicated proteins. *B*, Same as *A*, except that WM793 cells constitutively expressing NRAS^{Q61K} effector domain mutants were utilized. *C*, 1205Lu cells (parental, expressing NRAS$^{Q61K\ T35S}$, or expressing NRAS$^{Q61K\ E37G}$) were analyzed for EdU incorporation as in Fig. 1*A*. *D*, WM793 cells (parental, expressing NRAS$^{Q61K\ T35S}$, or expressing NRAS$^{Q61K\ E37G}$) were analyzed for annexin V staining in 3-D collagen as in Fig. 1*B*.

To test if association with RAF is required for mutant N-RAS to promote S-phase entry in the presence of PLX4720, EdU incorporation assays were performed on 1205LuTR cells expressing NRAS^{Q61K} effector domain mutations. In the presence of PLX4720, NRAS^{Q61K,T35S} promoted S-phase progression, whereas NRAS^{Q61K,E37G} was unable to promote S-phase progression in the presence of PLX4720 (Fig. 5*C*). We also examined resistance to apoptosis in the presence of PLX4720 using WM793 cells embedded in 3-D type-I collagen. WM793 NRAS^{Q61K,E37G}-expressing cells were susceptible to PLX4720-induced increases in annexin V staining, whereas N-RAS^{Q61K,T35S}-expressing cells displayed only a slight increase in annexin V in the presence of PLX4720 (Fig. 5*D*).

Consistent with prior studies, activation of the MEK-ERK1/2 pathway in parental WM793 cells requires BRAF but not CRAF (Fig. 6, *A* and *B*) (36). We next determined the involvement of RAF isoforms in MEK-ERK1/2 signaling in WM793-Res NRAS clonal cells. Compared with the control siRNA knockdowns, BRAF depletion decreased MEK and ERK1/2 phosphorylation in the absence and presence of PLX4720 in both clone 5 and clone 12 (Fig. 6*A*). By contrast, depletion of CRAF did not affect basal levels of phospho-MEK and phospho-ERK1/2 in the absence of PLX4720 but did inhibit MEK and ERK1/2 phosphorylation following PLX4720 treatment compared with control knockdowns (Fig. 6*B*). Together, these data indicate that WM793-Res NRAS cells primarily signal to MEK-ERK1/2 through BRAF in the absence of PLX4720 but utilize both CRAF and BRAF in the presence of PLX4720.

Depletion of SHOC2 Enables PLX4720-induced Inhibition of ERK1/2 and Apoptosis in PLX4720-resistant Cells—Scaffold proteins influence RAS-RAF signaling but how these molecules impact on the effect of RAF inhibitors remains poorly understood. We examined the role of the scaffold molecule, SHOC2/Sur8, which has been shown to bind to multiple RAS isoforms (13, 14, 37) and positively enhance growth factor and mutant RAS-induced signaling through the ERK1/2 pathway by promoting RAS-RAF association (14). SHOC2 depletion with multiple siRNAs did not alter levels of phosphorylated ERK1/2 in parental WM793 cells (Fig. 7*A*). In WM793-Res NRAS clones, basal ERK1/2 phosphorylation was not dramatically altered by knockdown of SHOC2 in the absence of PLX4720 but SHOC2 depletion did enhance the inhibition of ERK1/2 phosphorylation in PLX4720-treated WM793-Res NRAS clones (Fig. 7*B*). Effects were observed with multiple independent SHOC2 siRNAs (Fig. 7*B*) indicating that mutant NRAS signaling to MEK-ERK1/2 in PLX4720-resistant cells is partially dependent on SHOC2.

We next determined the requirement of SHOC2 on resistance to apoptosis induced by PLX4720. Control or SHOC2 knockdown WM793-Res NRAS cells were embedded in 3-D type I collagen in the presence of DMSO and PLX4720 for 48 h before annexin V analysis. Compared with control, SHOC2 knockdown did not lead to increased apoptosis in either clone 5 or clone 12 cells (Fig. 7*C*). However, the combination of SHOC2 knockdown and PLX4720 treatment resulted in a statistical increase in apoptosis (Fig. 7*C*). Since the levels of NRAS were altered with SHOC2 siRNA #1 (Fig. 7, *A* and *B*), we performed similar experiments with a second SHOC2 siRNA (#2) which

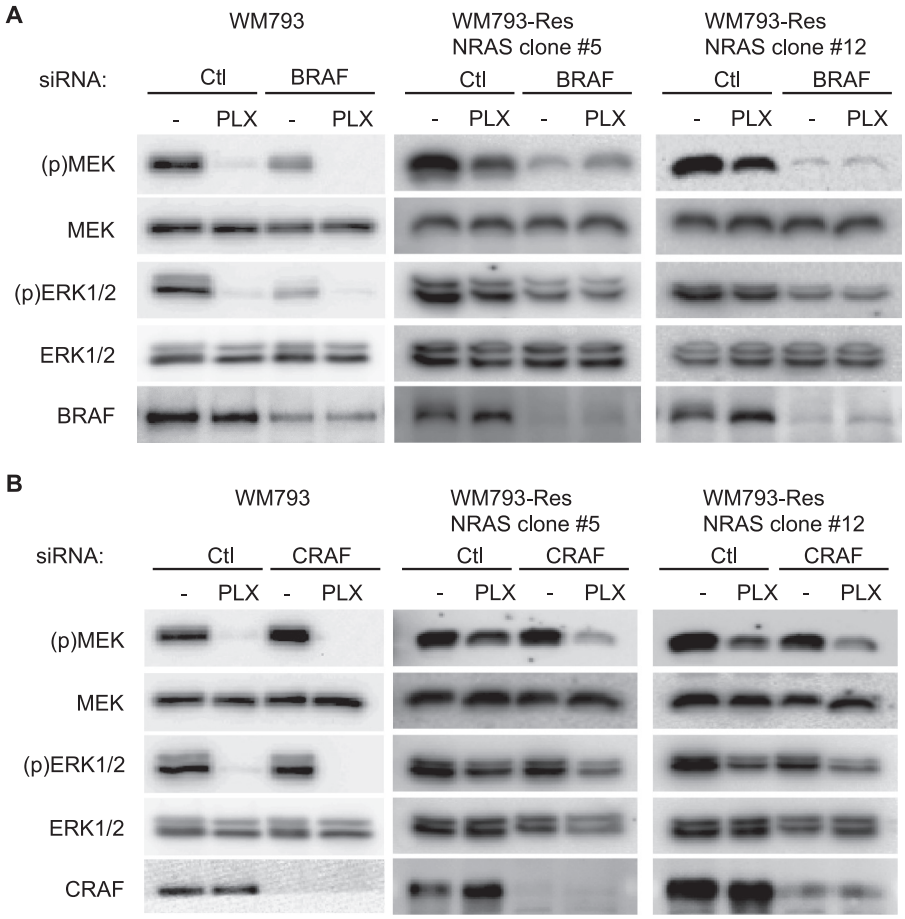

FIGURE 6. **ERK1/2 activity in WM793-Res NRAS cells is regulated by both BRAF and CRAF.** *A*, parental WM793 and WM793-Res NRAS cells (clones #5 and #12) were transfected with control (*Ctl*) or BRAF siRNA. Post-transfection, cells were treated with either DMSO (−) or 5 μM PLX4720 for 72 h with drug replenished for the last 24 h. Lysates were analyzed by Western blotting for phospho-MEK, total MEK, phospho-ERK1/2, total ERK1/2, and BRAF. *B*, as for *A*, except that cells were transfected with CRAF siRNAs and analyzed for CRAF expression.

did not affect NRAS levels. SHOC2 knockdown with siRNA #2 caused a small but consistent enhancement of apoptosis in WM793-Res NRAS cells in the presence of PLX4720 (Fig. 7*D*). This effect was further enhanced with NRAS co-knockdown. Together, these data indicate that NRAS is required for MEK-ERK1/2 signaling and resistance to RAF inhibitor-induced apoptosis in co-mutant $BRAF^{V600E}$/$NRAS^{Q61K}$ cells and that NRAS effects are likely mediated in part by SHOC2.

Because paradoxical signaling is induced by RAF inhibitors in cells with elevated RAS activity (19, 26–28, 38), we determined the involvement of SHOC2 in the paradoxical activation of MEK-ERK1/2 in mutant NRAS cells. As we have previously shown (26), treatment with PLX4720 enhanced phospho-MEK levels in mutant NRAS-harboring melanoma lines, WM1366 (Fig. 7*E*) and Sbcl2 (supplemental Fig. S2, *A* and *B*). Knockdown of NRAS or SHOC2 in these lines reduced both basal levels and PLX4720-induced phospho-MEK and phospho-ERK1/2 levels. Similar effects were observed by depleting SHOC2 with multiple independent siRNAs. Thus, MEK-ERK1/2 signaling in PLX4720-treated WM793-Res NRAS cells and parental mutant NRAS cells is regulated by SHOC2.

DISCUSSION

The RAF-MEK-ERK1/2 pathway is aberrantly activated in many human tumor types. In particular, the high frequency of activating BRAF mutations in melanomas, thyroid carcinomas, and colorectal tumors has focused attention on this serine-threonine kinase as a therapeutic target (15). Strong clinical efficacy has been shown with vemurafenib (PLX4032) in mutant BRAF melanoma patients leading to its FDA-approval (20–22). However, many tumors eventually progress, a process that is frequently associated with ERK1/2 re-activation (reviewed in Ref. 25). It is important to elucidate the intricate wiring of the ERK1/2 signaling pathway to understand the response to targeted therapies and, thus, the clinical use of RAF inhibitors.

From a screen for acquisition of resistance to PLX4720 (the tool compound for vemurafenib), we isolated cell lines co-expressing $BRAF^{V600E}$ and $NRAS^{Q61K}$. The latter alteration activates NRAS by reducing the rate of intrinsic GTP hydrolysis. This cell-based finding is consistent with initial findings from melanoma patients with acquired resistance to vemurafenib. Nazarian *et al.* described one patient (patient 55) with an isolated, left groin metastasis that initially shrank with vemurafenib treatment but subsequently re-grew (23). A Q61K NRAS mutation was detected in the re-growing tumor. The same patient developed additional nodal metastases, one of which was associated with a Q61R NRAS mutation. More recently, NRAS mutations were identified in 4 out of 19 samples

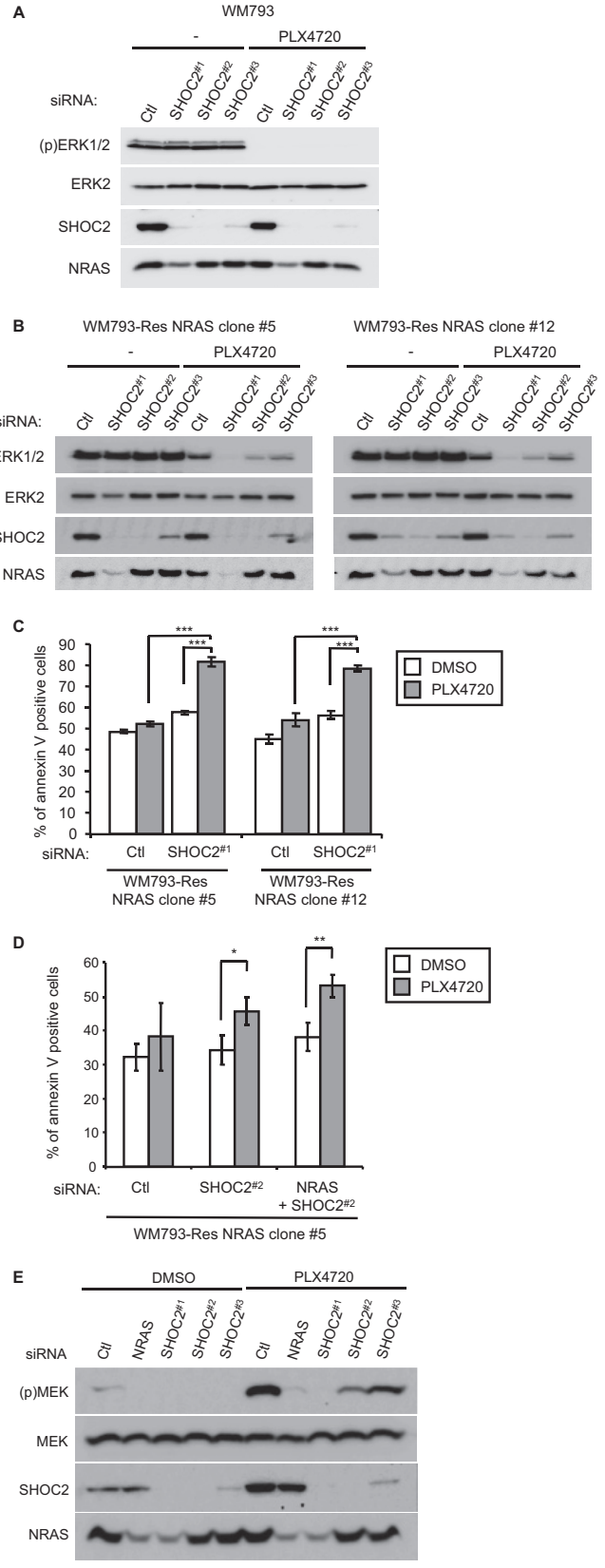

FIGURE 7. **The role of NRAS and SHOC2 in ERK1/2 activation and resistance to apoptosis in WM793-Res NRAS cells.** *A*, parental WM793 cells were transfected with control (*Ctl*) or SHOC2 (sequences #1, #2, and #3) siRNAs. Cells were treated with either DMSO (−) or 5 μM PLX4720 for 1 h. Lysates were analyzed by Western blotting for phospho-ERK1/2, total ERK2, SHOC2 and NRAS. *B*, as for *A*, except that WM793-Res NRAS cells (clones #5 and #12) were analyzed. *C*, WM793-Res NRAS cells (clone #5 and #12) were transfected with siRNA for control and SHOC2 (sequence #1). Post-transfection, cells were treated with DMSO and 1 μM PLX4720 for 48 h and annexin V analysis was performed. Assays were performed in triplicate. Error bars, S.D. ***, $p < 0.001$. *D*, same as *C*, except that WM793-Res NRAS clone #5 cells were transfected SHOC2 siRNA#2 and NRAS siRNA. Assays were performed in triplicate and apoptosis was quantitated using annexin V staining. Error bars, S.D. *, $p < 0.05$, **, $p < 0.01$. *E*, mutant NRAS melanoma cells, WM1366, were transfected with Ctl, NRAS, or SHOC2 (sequences #1, #2, #3) siRNA. Post-transfection, cells were treated with either DMSO (−) or 1 μM PLX4720 for 72 h (inhibitor was replenished for the last 24 h). Lysates were analyzed by Western blotting, as indicated.

from patients progressing on vemurafenib (29). The exact frequency of acquired NRAS mutations is currently being analyzed in larger patient cohorts at multiple centers. Nonetheless, these data underscore the patient relevance of mutations in NRAS associated with resistance to PLX4032.

How $NRAS^{Q61K}$ mediates resistance to RAF inhibitors remains unclear. PLX4720 inhibits cell cycle progression and enhances cell apoptosis in mutant BRAF melanoma cells (17, 34). Co-expression of $NRAS^{Q61K}$ negates the inhibitory effects of PLX4720 on entry into S phase and PLX4720-initiated apoptosis in mutant BRAF melanoma lines. These effects are associated with the inability of PLX4720 (and vemurafenib) to inhibit MEK and ERK1/2 activation. Recent studies on KRAS signaling indicate that distinct activating mutations and even amino acid substitutions may mediate differential signaling and response to chemotherapeutics (33). Multiple NRAS Q61 substitutions have been identified in melanoma. Our studies herein show that Q61H, Q61R, and Q61L substitutions in NRAS were all sufficient and equivalent in their ability to promote ERK1/2 reactivation. Thus, multiple mutations in NRAS at codon 61 are able to change the response of mutant BRAF harboring cells to RAF inhibitors.

We further investigated the mechanism underlying mutant NRAS-mediated resistance. Effector domain studies show a requirement for the RAF binding site in mutant NRAS in the bypass of PLX4720 inhibitory effects. Our knockdown data suggest that PLX4720 causes a switching of the requirement for RAF isoforms in resistant cells harboring $NRAS^{Q61K}$. In the absence of PLX4720, signaling to MEK occurs via BRAF and is independent of CRAF. This BRAF dependence is similar to that observed in the parental cells (39, 40). By contrast, in $NRAS^{Q61K}$ resistant cells treated with PLX4720, activation of MEK-ERK1/2 require both BRAF and CRAF. The altered requirement of RAF isoforms upon expression of mutant NRAS is consistent with published data showing that mutant, active HRAS induces heterodimerization of BRAF with CRAF (41). This condition is also similar to the mechanism underlying paradoxical activation of the ERK1/2 in which RAF inhibitors hyperactivate the pathway in cells with elevated RAS activity via drug inactivated BRAF binding to and trans-activating CRAF (26, 28, 38). Knockdown of CRAF alone was not sufficient to inhibit entry into S phase in the WM793-Res NRAS cells since the requirement is partial (data not shown). This is likely due to flexible switching between all RAF isoforms during resistance to RAF inhibitors (42).

Signaling through the ERK1/2 pathway is fine-tuned by scaffold proteins; however, the extent that scaffolding regulates the

response to targeted therapies is poorly described. We investigated the RAS-RAF binding protein, SHOC2. Although reports on SHOC2 have yielded differing conclusions regarding the RAS isoforms to which SHOC2 binds (13, 14, 37), they agree that SHOC2 positively enhances growth factor and mutant RAS-induced signaling through the MEK-ERK1/2 pathway by promoting RAS-RAF association. We show that SHOC2 is not required for activation of ERK1/2 when signaling through mutant BRAF is dominant, *i.e.* parental mutant BRAF melanoma cells or resistant mutant NRAS-expressing cells in the absence of PLX4720. By contrast, our initial data suggest that SHOC2 modulates ERK1/2 activation when a CRAF-dependent mechanism is dominant such as in resistant $BRAF^{V600E}$/$NRAS^{Q61K}$ treated with PLX4720 or parental mutant NRAS melanoma cells. This is consistent with a previous report showing a requirement for SHOC2 in MEK-ERK1/2 activation of a mutant NRAS cell line and the G463V BRAF/mutant KRAS harboring MDA-MB-231 cells (37). G463V BRAF has intermediate kinase activity and is likely to act via binding to CRAF (16). Overall, these findings suggest that acquired mutation of NRAS re-wires RAS-RAF signaling in response to RAF inhibitor.

In summary, we describe melanoma cells with resistance to a clinically relevant RAF inhibitor that is associated with a mutation in NRAS. We provide evidence that these mutant NRAS resistant cells alter their signaling connections in response to RAF inhibitor resulting in a shift to dependence on CRAF and a role for the RAS-RAF scaffold molecule, SHOC2.

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